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HISTOCHEMICAL STUDIES OF AMINOPEPTIDASE BY MEANS OF L-N(5-BROMOINDOL-3-YL) LEUCINAMIDE

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DEPARTMENT OF THE ARMY
Fort Detrick
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HISTOCHEMICAL STUDIES OF AMINOPEPTIDASE BY MEANS OF L-N(5-BROMOINDOL-3-YL)LEUCINAMIDE

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MEDICAL SCIENCES LABORATORY

Project 1T013001A91A

April 1968

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ABSTRACT

Aminopeptidase in tissue and component cells thereof can be demonstrated by a new synthetic indolyl substrate with leucine attached to a halogen-substituted indolyl moiety. The tissue enzyme hydrolyzes the peptide bond adjacent to the free amino group to form a chromogenic bisindigo at enzymic sites within the cells and tissues. Evidence of high enzyme activity was seen in the kidney, parathyroid gland, and connective tissue cells of the lamina propria. The enzyme activity in the kidney was most intense in the lower cortex; the activity of the upper cortex was about one-half. In the upper cortex, the enzyme was present mainly in the basilar region of the cell, whereas, in the lower cortex, it was more intense in the luminal portion. The medulla of the kidney showed only a slight reaction. Conspicuous and high enzyme activity was seen in certain connective tissue cells of the lamina propria. These cells tend to exhibit certain fibrillary processes. The stroma of the endometrium of the uterus revealed cells with high activity. These cells have an elongated cytoplasm with a dense nucleus and are morphologically similar to other stromal cells in the proliferative stage of the endometrium. The parathyroid gland showed a strong enzyme reaction that was uniformly distributed throughout the cells of the gland. The thyroid gland showed a medium enzyme activity, seen as granules in the follicular epithelial cells. The liver showed a medium activity. The thymus showed activity mainly in the reticuloendothelial cells. There was a great deal of variation in enzyme activity that seemed to depend upon physiological states. Thus, in the ovary, there was marked activity in the atretic follicle. Pulmonary alveolar macrophages showed medium to strong activity.

I. INTRODUCTION

Spackman, Smith, and Brown[1] isolated a highly purified aminopeptidase from the swine kidney that was shown by Smith and Spackman[2] to be consistent with the properties of the enzyme isolated from the mucosa of the intestine. Although this enzyme was referred to as leucine aminopeptidase because of the effective and superior hydrolysis of L-leucinamide, it was obvious that a number of other amino acid substrates also were hydrolyzed by this purified enzyme,[2] but to a lesser degree. The purified enzyme was found to be a metalloenzyme requiring Mn^{++} or Mg^{++}. The former metal was a better activator, but the latter was preferable as it stabilized the enzyme better. The D-leucinamide gave no reaction even with the addition of Mn^{++} or Mg^{++}.

We have used a halogenated indolyl substrate containing the DL-leucine hydrobromide moiety to demonstrate aminopeptidase in tissue.[3] The enzyme hydrolyzed the leucine moiety adjacent to the free amino group to form the final reaction product, an insoluble indigo. The structural formula of this more recently described compound, L-N(5-bromoindol-3-yl)leucinamide hydrochloride, is shown in Figure 1. Our purpose here is to describe the histochemical appearance and localization of the enzyme with this substrate.

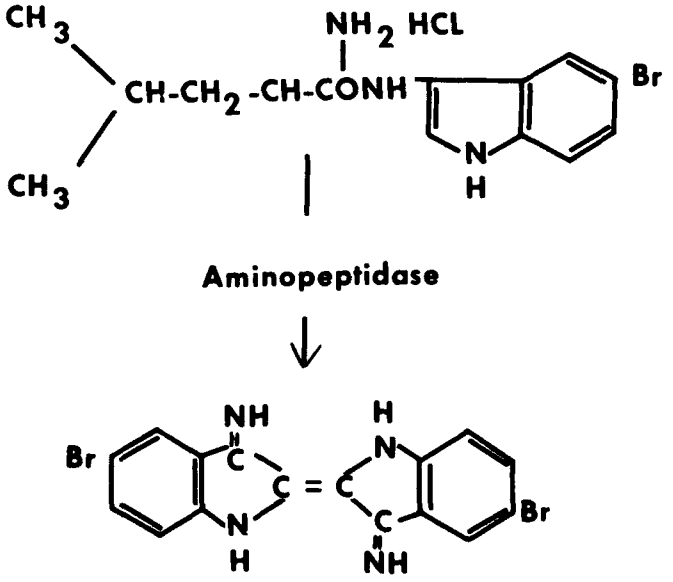

Figure 1. L-N(5-bromoindol-3-yl)leucinamide Hydrochloride Hydrolyzed by Tissue Aminopeptidase to Form the Brom-Substituted Indigo that Indicates Sites of Enzyme Activity.

II. MATERIALS AND METHODS

Tissues from rabbits, mice, rats, and guinea pigs were used for this study. Fresh-frozen cryostat-cut tissue was incubated in a solution containing both the L and the DL forms of indolyl substrate. Single cells from alveolar monocytes harvested from the rabbit and affixed to Mylar strips or glass slides were also studied. The components of the substrate solution have been previously described.[3] Attempts were made to demonstrate tissues of relatively high, medium, or low enzyme activity.

III. RESULTS

The kidney gave the most intense reaction. This was apparent in the cells of the distal convoluted tubules adjacent to the medulla in the lower cortex (Fig. 2). The distribution of the enzyme in this area was most intense in its luminal portion (Fig. 3). The upper cortex showed a lesser enzyme intensity and had a basilar distribution, mainly in the proximal convoluted tubules. While the enzyme was evenly distributed in the lower cortex, its distribution varied considerably from tubule to tubule in the upper cortex (Fig. 4).

Marked enzyme reaction was seen in the cells of the lamina propria of the small intestine. The majority of these cells were identified as fibroblasts (Fig. 5). The cells of the mucous membrane showed a lesser reaction. Another tissue showing strong enzyme reaction was the stroma of the endometrium, particularly in its proliferative phase. The involved cells were elongated and fibroblastic in appearance (Fig. 6) and not unlike the enzyme-negative cells that compose the endometrial stromal population. Intense activity observed in the atretic ovum was directly proportional to the stage of dissolution.

Among tissues that showed an enzymatic reaction of medium intensity was the parathyroid gland. The exact cell involved could not entirely be determined. The thyroid gland showed a medium enzyme reaction involving the epithelial cells lining the acini (Fig. 7). The thymus showed a reaction that was mainly confined to the cytoplasm of the reticulum cells, the Hassall's corpuscles being negative. The pancreas, adrenals, and skin showed a low activity under normal conditions.

Some extreme variations in enzyme intensity occurred, particularly in the lymphatic system. Under well-controlled conditions, alveolar mononuclear cells showed a medium activity (Fig. 8). This activity was increased by subjecting the animal to BCG. The spleen and lymph nodes varied in enzyme intensity, especially in large reticular cells.

Figure 2. Lower Cortical Section of Kidney Showing Uniform Aminopeptidase Reaction in Distal Convoluted Tubules. Lower left shows faint enzyme reaction in medulla of kidney. Cryostat-cut section incubated in substrate. Not counterstained. 90X.

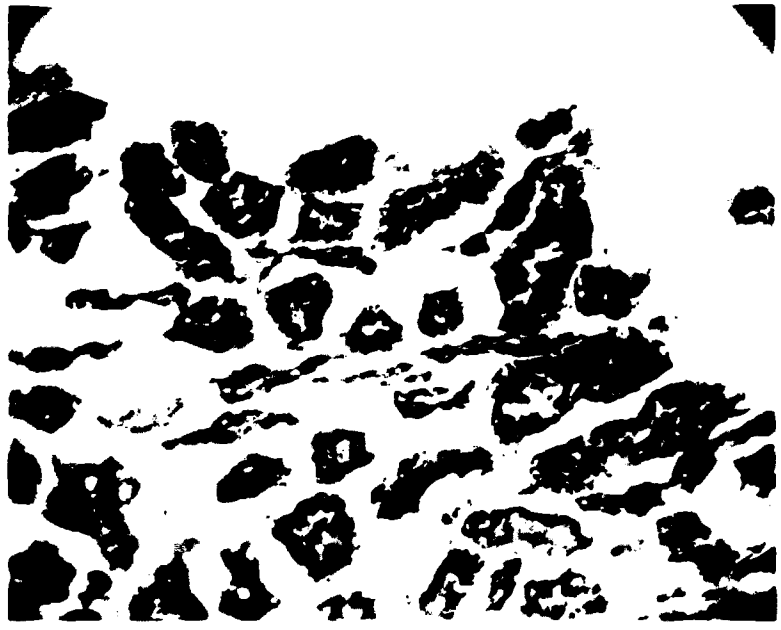

Figure 3. Section of Lower Kidney Cortex Showing Intense Aminopeptidase Reaction Concentrated in Luminal Borders of Tubules. Cryostat section. Not counterstained. 280X.

Figure 4. Section of Upper Cortex of Kidney Showing Two Unreactive Glomeruli on Lower Right. Some but not all of proximal convoluted tubules show aminopeptidase reaction, which is mainly concentrated in basilar portion of cells. Not counterstained. 280X.

Figure 5. Section of Ileum Showing Intestinal Villi. Intense aminopeptidase reaction occurs in certain cells of lamina propria. Most of these can be identified as fibroblasts. Epithelial cells of mucosa show faint enzyme reaction. Not counterstained. 135X.

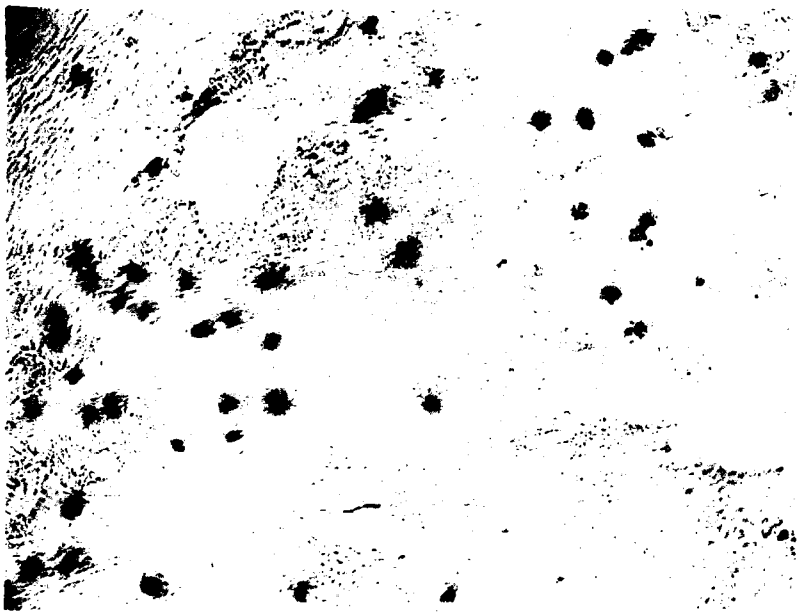

Figure 6. Section of Endometrium in the Proliferating Stage Showing Intense Aminopeptidase Reaction in Certain Cells of Endometrial Stroma. Upper left shows a positive fibroblast in the uterine wall. Not counterstained. 280X.

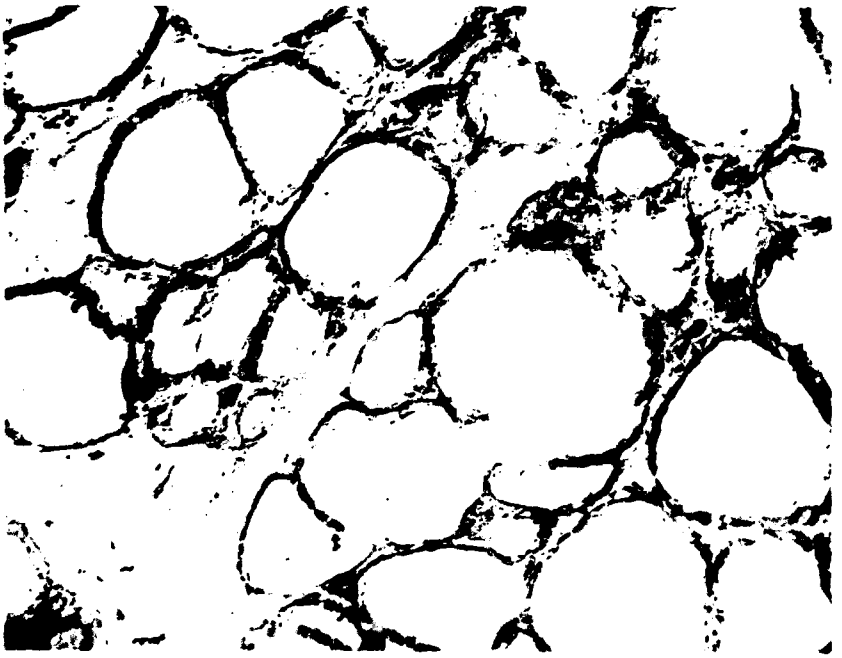

Figure 7. Section of Thyroid Gland Showing Moderate Aminopeptidase Reaction of Epithelial Cells of Thyroid Follicles. The colloid is negative. Not counterstained. 200X.

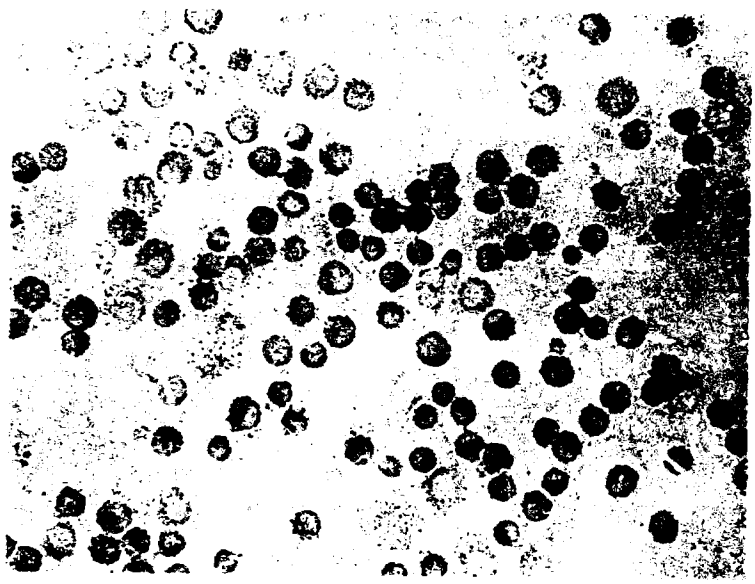

Figure 8. Harvested Alveolar Monocytes Showing Aminopeptidase Reaction in Cytoplasm. Not counterstained. 400X.

IV. DISCUSSION

A new substrate has been introduced and adapted to demonstrate aminopeptidases in tissues and cells. This histochemical compound, L-N(5-bromoindol-3-yl)leucinamide hydrochloride, is similar in principle to the recently developed indolyl compounds introduced by us for the detection of β-galactosidase,[4] β-glucuronidase,[5] and β-fucosidase.[6] It differs from the previous DL form of indolyl leucinamide, which contains the inactive D moiety, and is in the form of a hydrochloride instead of a hydrobromide. The aminopeptidases in tissues and cells hydrolyze the peptide bond of α-amino acids adjacent to a free amine, resulting in a bisindigo at enzymic sites. The conditions are consistent with the findings of Smith and Spackman[3] in that the pH optimum ranged from 7.5 to 8 and that the best results were obtained by adding Mn^{++} or Mg^{++} ions. Preferential hydrolytic activity depends upon the L-leucinamide, although other substrates are hydrolyzed but to a far lesser extent. The D-leucinamide is not hydrolyzed. Previous work by Burstone and Folk[7] and by Nachlas, Crawford, and Seligman[8] has shown the feasibility of histochemical demonstration of aminopeptidases in tissues. The type of reaction was represented by an azo dye coupling procedure employing L-leucyl-β-naphthylamide that released β-naphthylamide. Burstone and Folk found the garnet GBC salt to be a preferential coupler; later, Nachlas et al.[9] using the L-leucyl-4-methoxy-β-naphthylamide with Diazo Blue B as a coupler, improved their original method. Our substrates, both the DL bromide and particularly the L-N(5-bromoindol-3-yl)leucinamide chloride, have the added advantages of not requiring a coupler and of forming reaction products that are stable.

It is obvious that aminopeptidases are a heterogeneous group in tissues, which may explain some of the variations observed in comparing our results with those of the previous investigators. The conditions under which we use our substrates and the results are consistent with those of Smith and Spackman,[3] who defined aminopeptidases in terms of Mn^{++} and Mg^{++} requirements as well as high pH optima and preferential leucinamide hydrolysis. This is not entirely possible with azo dye methods, as they require a low pH for hydrolysis, thus not complying with the strict requirements of the purified enzyme.

In view of the apparent heterogeneity of aminopeptidases and some differences in comparing results of methodology, difficulties arise in being able to classify high, medium, and low enzyme activity in tissue by histochemical means. However, we did find that high activity was present in the kidney, parathyroid, fibroblasts of the intestinal lamina propria, and endometrial stroma; medium activity in the thymus, thyroid, and liver, and low activity in pancreas, adrenal, and skin. In addition, and probably most important, was the extreme variability, particularly in lymphatic tissue (spleen and lymph node) and in ovary. The latter was

emphasized by Hopsu, Riekkinen, and Luostarinen[10] in referring to the hormonal influences on aminopeptidases in the accessory reproductive tract. We found a very striking relationship between the atretic, blighted ovum and aminopeptidase activity that may be consistent with hydrolytic destruction. We have also observed an increase in the enzyme in alveolar monocytes that are subject to stimulatory effects. Variations in the lymphatic system were also seen in pathologic conditions.

V. SUMMARY

A new substrate for the histochemical demonstration of tissue aminopeptidases has been described. This compound is L-N(5-bromoindol-3-yl)-leucinamide hydrochloride. It does not contain the inactive D form of indolyl hydrobromide previously described by us. Both isomers, however, are active for aminopeptidase, although the L indolyl hydrochloride is preferable. On hydrolysis by tissue aminopeptidases, it forms a bisindigo at enzymic sites. It needs no additional coupling reagent. The condition under which it operates is consistent with the strict definition of the highly purified aminopeptidase.

The aminopeptidases are generally high in kidney, fibroblasts of the intestinal lamina propria, parathyroid, and uterus. Variability can be demonstrated caused by pathologic and physiological states as indicated by follicular atresia and dissolution of the blighted ovum as well as by changes in monocytes and cells in the lymphatic system.

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